IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent application of Misa OCHIIAI et al.	:	Confirmation No.: 1234
Serial No.:	10/594,772	:	Group Art Unit: 1635
Filed:	July 6, 2007	:	Examiner: Richard A. Schnizer
For:	BREEDING METHOD OF LIPID PRODUCING FUNGI AND USE OF SUCH METHOD	:	

DECLARATION UNDER 37 C.F.R. §1.132 OF MASAKO FUKUSHI-MIZUTANI

Mail Stop AMENDMENT
Commissioner for Patents
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Sir:

I, Masako Fukuchi-Mizutani, declare and state as follows:

1. I reside at 53-60 Katsurainui-cho, Nishikyo-ku, Kyoto, Kyoto 615-8086 Japan.

2. I have been employed by Suntory Holdings Limited since 1991 to the present date.

3. It is my understanding that this application is assigned to Suntory Holdings Limited.

4. I am not a listed inventor on this application.

5. I have a doctorate degree in Agriculture, received in 1999 from the Kyoto University.

6. I have more than 10 years of experience in the area of plant and fungi fatty acid metabolism. My area of expertise includes manipulation of fatty acid metabolism using fatty acid desaturases and fatty acid elongases from plants or fungi.

7. I have reviewed the Advisory Action mailed August 12, 2009 and the Office Action mailed April 7, 2009.

8. It is my understanding that the claims remain rejected as obvious over (a) **Certik** et al. [*Trends in Biotechnology* 16(12): 500-505, 1998], (b) **Ueda** (*J. Neurogenetics,* 15(3-4): 193-204, 2001], and (c) **Mackenzie** et al., [*App. Env. Microbiol* 66(1): 4655-4661, 2000]. I have reviewed these references and make the following comments thereon.

9. These references do not suggest a breeding method of lipid producing fungi for *Mortierella* comprising suppressing expression of a lipid metabolism gene that encodes an enzyme selected from GLELO, MAELO, Δ5 fatty acid desaturase, Δ6 fatty acid desaturase, and Δ12 fatty acid desaturase in the lipid producing fungi, wherein the expression suppressing step includes an RNAi step of suppressing expression of the lipid metabolism gene by an RNAi method, or a co-suppression step of suppressing expression of the lipid metabolism gene by a co-suppression method for the following reasons. **Certik** et al. may disclose that certain randomly produced mutants of *M. alpina*, which are rendered completely defective in certain enzymes of the lipid synthesis show an effect in the synthesis of lipids. However, it can in no way be derived from Certik that by using the RNAi approach a control and thereby a fine tuning of the expression/suppression level of lipid synthesis genes would be possible thereby allowing to design *Mortierella* strains producing a desired pattern and composition of fatty acids. **Mackenzie** et al only describe that *M. alpina* was transformed successfully to hygromycin B resistance by using a homologous histone H4 promoter to drive gene expression and a homologous ribosomal DNA region to promote chromosomal integration (see, for example, Abstract at page 4655). This reference does not teach nor even suggest successful suppression of a specific gene with use of RNAi or co-suppression technique in *M. alpina*. **Ueda** generally describes that RNAi provides a means of selectively inhibiting expression of genes of choice, but fails to teach or suggest that RNAi approach can be effective in suppressing the claimed target genes, i.e., GLELO, MAELO, Δ5 fatty acid desaturase, Δ6 fatty acid desaturase, and Δ12 fatty acid desaturase in the lipid producing fungi. Accordingly, I believe that it was difficult to combine the teachings of these references to arrive at the present invention, given what was known at the time and the post-filing references, a person in this field would not have put these elements together and had an expectation that it would work as it does.

REFERENCES OF PROUDFOOT AND NAKAYASHIKI

10. At the priority date of this application, I believed that it was unobvious to use the RNAi approach or the co-suppression approach in *Mortierella* in order to try to regulate the production of lipids, in particular PUFAs. It was not known as to whether the RNAi approach or the co-suppression approach would work in lipid producing *Mortierella*. The references of Proudfoot et al., 131 CELL 649 (2007) and Nakayashiki et al., 11 CURR. OPIN. MICROBIOL. 494 (2008) were published after the filing date of this application. These references support my position as mentioned above.

11. Proudfoot discusses RNA interference in fungi. For example, Proudfoot reports, at the left column, lines 2-6 from the bottom at page 498, that in *Ustilago maydis* (in addition to *S. cerevisiae*), the entire RNA silencing machinery appears to be lost. This paper further describes that in *Ustilago hordei*, a close relative to *U. maydis*, RNA silencing was demonstrated. Thus the loss of the RNA silencing machinery seems to sporadically occur in the fungi kingdom (*see* the left column, last line to right column, line 9 at page 498).

12. In conclusion, although these papers were published after the priority date of this application, I believe that these papers can show that a skilled person in the art was not particularly motivated to try RNAi approach or co-suppression approach in gene silencing in fungi even after the filing date.

DATA

13. The present application shows in Example B and C for MAELO and Δ12 fatty acid desaturase that their suppression via RNAi leads to the desired effects. Moreover, the results of Experiments 1 to 3 described below show that the suppression of Δ5 fatty acid desaturase, Δ12 fatty acid desaturase, or GLELO in *Mortierella* by co-suppression improved the synthesis of PUFAs. Finally, Experiment 4 shows the suppression of Δ6 fatty acid desaturase by co-suppression leads to statistically significantly improved effects for fatty acid synthesis.

Experiment 1 (Co-suppression of Δ5 fatty acid desaturase gene)

14. A high expression vector for Δ5 fatty acid desaturase gene was constructed as follows. With use of cDNA of *M. alpina* 1S-4 strain as a template, the target fragment was amplified with the forward primer MAD5-F: TCTAGAATGGGTGCGGACACAGGAAAAACC and the reverse primer MAD5-R: AAGCTTTTACTCTTCCTTGGGACGAAGACC. The fragment amplified was digested with restriction enzymes XbaI and HindIII, respectively. Then, the fragment obtained was inserted into the prescribed site in the multi-cloning sites of pDura5MCS, to provide plasmid pDura5-Δ5.

15. Urasil auxotrophic strain #2-5 derived from *M. alpina* 1S-4 was transformed with the plasmid pDura5-Δ5. Twenty-four strains of the transformants were cultured for 7 days in 4 ml of GY(2:1)(medium comprising glucose 2%, yeast extract 1%), and then the fatty acid compositions were analyzed. Compared with the wild type, several strains demonstrated higher productivity of arachidonic acid. Interestingly, one (#14) of the strains analyzed showed that the production of arachidonic acid was significantly reduced, whereas the precursor form of arachidonic acid, DGLA, was accumulated (Table 1). No significant differences were seen in yeast cell concentration and total fatty acid production between this strain and those of the wild type. Therefore, it is supposed that strain #14 adversely suppressed the function of Δ5 fatty acid desaturase gene, which was expected to be constitutively expressed. Thus, the precursor form, DGLA, was accumulated. In conclusion, it becomes clear that co-suppression occurs in *M. alpina*.

16. It should be noted, that the level of precursor, DGLA, is not something that it would have been capable of prediction as to what level they would accumulate.

Table 1: Fatty acid compositions of Δ5 fatty acid desaturase gene-highly expressed strains

	Δ5-transformant		Control	
	#1	#14	1S-4	C-10
fatty acid composition (%)				
16:0	11.89	20.1	13.96	15.48
18:0	6.37	7.76	6.53	6.82
18:1	10.32	14.78	11.69	13.7
18:2	6.92	3.86	8.31	7.41
18:3(n-6)	2.93	3.6	3.26	2.75
DGLA	3.97	35.31	3.6	2.81
AA	48.07	6.57	44.8	41.36
24:0	6.47	5.04	5.27	6.75
Others	3.05	2.97	2.58	2.92
fungus conc.(g/L)	11.97	11.02	12.35	12.42
fatty acid productivity(g/L)	2.71	2.86	2.95	2.91
lipid contents within fungus (%)	22.66	25.98	23.88	23.44
AA productivity(g/L)	1.30	0.19	1.32	1.20

#1: Strain showing high production of arachidonic acid; #14: Expected to accumulate DGLA by co-suppression. C-10: pDura5 transfectants

Experiment 2 (Co-suppression of Δ12 fatty acid desaturase gene)

17. A high expression vector for Δ12 fatty acid desaturase gene was constructed as follows. With use of cDNA of *M. alpina* 1S-4 strain as a template, the target fragment was amplified by PCR with the forward primer MAD12-F: TCTAGAATGGCACCTCCCAACACTATTG and the reverse primer MAD12-R: AAGCTTTTACTTCTTGAAAAAGACCACGTC. The amplified fragment was digested with restriction enzymes XbaI and HindIII, respectively. The amplified fragment was inserted into the prescribed site of the vector pDuraSC, to provide plasmid pDuraSC-Δ12.

18. Urasil auxotrophic strain #2-5 derived from *M. alpina* 1S-4 was transformed with the plasmid pDuraSC-Δ12. 112 strains of the resultant transformants were cultured with shaking for 4 days at 28°C in 4ml of GY(2:1). Then, the fatty acid compositions of the yeast cells were analyzed. Sixteen out of the 112 strains showed that compared with the wild type, the production of oleic acid (substrate for Δ12 fatty acid desaturase) was increased. Also, n-9 fatty acids, 18:2(n-9), 20:2(n-9) and Mead acid (20:3(n-9)) were produced. The data suggest that expressional suppression of Δ12 fatty acid desaturase gene

occurred by co-suppression. Two strains, Δ12-4 and Δ12-9 strains, were considered to show co-suppression effects. The two strains and the wild type strain were cultured with shaking for 8 days at 28°C in 4 ml of GY(2:1), supplemented with 2% glucose on day 3 after start of cultivation. Then the fatty acid compositions of the yeast cells were analyzed. The results are as shown in the following Table 2.

Table 2: Fatty acid compositions of Δ12 fatty acid desaturase gene-highly expressed strains

Strain	Δ12-4	Δ12-9	1S-4
16:0	11.77	18.34	13.66
18:0	6.73	5.24	7.20
18:1	41.28	25.02	7.97
18:2(n-9)	9.03	0.83	0.00
18:2(n-6)	1.35	11.16	7.57
18:3(n-6)	0.85	2.70	3.49
20:0	0.62	0.48	0.64
20:1	2.06	1.13	0.00
20:2	0.91	0.00	0.00
20:3 (n-9)	7.70	0.64	0.00
20:3 (n-6)	0.69	2.26	3.80
22:0	1.86	1.37	1.88
20:4 (n-6)	7.42	15.42	46.48
24:0	3.62	2.33	3.05
Other	4.11	13.10	4.26
Total	100.00	100.00	100.00
n-9 relatives, total	58.92	26.49	7.97
n-6 relatives, total	10.31	31.53	61.34

19. Among the strains showing co-suppression effects, the suppression level of gene expression varied from strain to strain. It is possible to select desired strains by considering suppression levels of each strain.

Experiment 3 (Co-suppression of GLELO Gene)

20. A high expression vector for GLELO gene was constructed as follows. With use of cDNA prepared from *M. alpina* 1S-4 strain as a template. The GLELO cDNA fragment was amplified by PCR with the primer GLELO-5: GTCTAGAATGGAGTCGATTGCGCAATT and the primer GLELO-6: GGAGCTCTTACTGCAACTTCCTTGCCTTC. The amplified cDNA fragment of about 1

kbp was cloned using TOPO-TA-cloning Kit Invitrogen. A cloned plasmid having a correct nucleotide sequence was named pCR-GLELO.

21. The plasmid pDUraSC was digested with restriction enzyme XhoI. The ends of the obtained fragment were blunted with Blunting Kit (Takara Bio Inc.), followed by further digestion with restriction enzyme, XbaI. The plasmid pCR-GLELO was digested with restriction enzyme SacI. The ends of the obtained fragment were blunted with the Blunting Kit (Takara Bio Inc.), followed by further digestion with restriction enzyme, XbaI. The obtained fragments were ligated with Ligation High (Toyobo Co.,Ltd.), to provide plasmid pDuraSC-GLELO.

22. Urasil auxotrophic strain #2-5 derived from *M. alpina* 1S-4 was transformed with the plasmid pDuraSC-GLELO. Thirty-four strains of the resultant transformants were inoculated in 4 ml of GY(2:1) and cultured with shaking at 28°C. On day 3, the cultured medium was supplemented with 2% glucose. On day 7, the yeast cells were collected to analyze the fatty acid compositions. As a result, two strains (#2 and #12) out of the 34 strains obtained, showed that compared with the wild type, the content of gamma-linolenic acid (substrate for GLELO protein) was increased. This suggests that expressional suppression of GLELO gene occurred by co-suppression (see Table 3).

Table 3: Fatty acid compositions of yeast cells

	transformant		
%	#2	#12	1S-4
16:0	13.59	12.73	11.72
18:0	7.05	6.94	7.11
18:1	12.98	9.61	9.05
18:2	11.25	11.31	6.98
18:3(n-6)	20.17	24.36	3.05
DGLA	2.08	2.05	4.27
ARA	21.74	22.58	46.06
24:0	3.70	4.18	5.04
Other	7.45	6.25	6.71
Total	100.00	100.00	100.00

Experiment 4 (Co-suppression of Δ6 fatty acid desaturase)

23. A high expression vector for Δ6 fatty acid desaturase I gene (Δ6I; GenBank Accession No. AB020032) was constructed as follows. Using cDNA of *M. alpina* 1S-4 strain as a template. The target cDNA fragment (cDNA of Δ6I) was amplified by ExTaq PCR with a primer MAD6I-F: TCTAGAATGGCTGCTGCTCCCAGTGTGAGG and a primer MAD6I-R: AAGCTTTTACTGTGCCTTGCCCATCTTGG. The amplified fragment of about 1.4 kbp was cloned with TOPO-TA cloning kit (Invitrogen). A plasmid having a correct nucleotide sequence was designated as Plasmid pCR-Δ6DSI.

24. Plasmid pCR-Δ6DSI and Plasmid pDUraSC were digested with restriction enzymes XbaI and HindIII respectively. The digested fragment obtained from Plasmid pDUraSC. A 1.4 kbp fragment was obtained from Plasmid pCR-Δ6DSI, and was ligated to construct Plasmid pDuraSC-Δ6DSI.

25. In 3 strains out of the 42 strains (#1, #7, and #41) obtained, production of linolenic acid (a substrate of Δ6 fatty acid desaturase I) was increased in a statistically significant amount (*i.e.*, greater than 20%) as compared to that produced by the wild-type. *See* Table below. It was confirmed that expression of Δ6 fatty acid desaturase was suppressed by co-suppression.

	Transformant			Wild-Type
	#1	#7	#41	1S-4
16:0	14.19	14.58	12.99	15.03
18:0	11.09	10/60	12.02	9.26
18:1	16.23	16.20	17.74	17.19
18:2(n-6)	26.86	24.45	25.72	7.46
18:3(n-6)	1.46	1.70	1.49	3.21
DGLA	1.35	1.80	1.49	4.45
ARA	11.74	15.07	11.89	31.44
24:0	4.29	3.70	3.86	3.65
Other	12.78	11.90	12.79	8.31
Total	100.00	100.00	100.00	100.00

26. As mentioned above, it was unexpected that a co-suppression method could be effective in suppression of the three genes encoding desaturases. However, as apparent from the above results, all three desaturase genes were capable of co-suppression. The facts that the Δ6 fatty acid desaturase gene was successfully suppressed by the co-suppression method, was unexpected, because there are two genes in *Mortierella*, and it was unexpected that both genes could be co-suppressed using a construct based on the sequence of only one of the two genes. Also unexpected was that the amount of the substrate, 18:2(n-6), was increased at least 2X as compared to the control. Also, production of direct or indirect products produced by the action of the Δ6 fatty acid desaturase suppression (i.e., 18:3(n-6), DGLA and ARA) was reduced by more than 50% as compared to the controls

CONCLUSION

27. Based on the data above, the references of Proudfoot and Nakayashiki, and what was taught in the references relied upon by the Office, there would have been no expectation at the time that co-suppression of any one of the genes would have been reasonably expected to yield the results exemplified.

(SIGNATURE PAGE FOLLOWS)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

September 7th, 2009.
Date

Masako Fukuchi-Mizutani